DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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CODON 215 MUTATION PCR DETECTION ASSAY FOR HIV RNA

I. PRINCIPLE

The Codon 215 Test is a polymerase chain reaction (PCR)-based test designed to detect the presence of a specific DNA sequence at Codon 215 in the reverse transcriptase (RT) gene of human immunodeficiency virus (HIV). HIV isolates possessing a DNA sequence 215 of TAC or TTC at codon 215 show reduced susceptibility to zidovudine. Clinical studies have indicated that patients with HIV-1 possessing these "mutant" sequences have a greater risk for clinical progression than patients with the "wildtype" sequence (ACC).

In this test, clinical samples are processed and a portion of the HIV RT gene is amplified by PCR. A second, selective round of two PCR reactions is then conducted in parallel using PCR product from the first reaction: one reaction will amplify wildtype codon 215 sequences, and one reaction will amplify mutant sequences (if any) at that position. Both second round reactions are then run on an agarose gel to determine which second round successfully amplified, indicating the genotype of the virus tested reaction (wildtype or mutant).

II. SPECIMEN REQUIREMENTS

Serum, plasma, and seminal fluid are suitable specimens. Aliquots of 0.5 to 1.0 mL should be stored at -70^{0}C or lower. Plasma isolated from heparinized blood is unreliable for PCR tests, and should be avoided. Suitable anticoagulants are citrate, acid citrate dextrose, and EDTA.

III. REAGENTS

A. Reagents Unique for RNA Preparation Method A:

Solution D: A solution containing 5M guanidine thiocyanate, 0.033M sodium citrate (pH 7.0), 10% sarcosyl, and 0.007M 2-mercaptoethanol, stored at room temperature.

Sodium Acetate: A 3M solution (pH 4.5), stored at room temperature.

Yeast Transfer RNA, 100 µg/mL. Aliquots should be stored at -20^{0}C.

Phenol: Tris-saturated phenol (pH 8.0), stored at 4^{0}C.

Chloroform:Isoamyl alcohol: A 49:1 mixture of chloroform and isoamyl alcohol, stored at room temperature.

B. Reagents Unique for RNA Preparation Method B:

Tri-Reagent (Molecular Research Centers, Inc., Cincinnati, OH), stored at 4^0C.

Chloroform, stored at room temperature.

C. Common Reagents

Viral Lysis Buffer: A solution containing 2 mm dithiothreitol, 0.1% Noniodet P-40, 0.01% RNAsin (or equivalent), and 0.04 µg/mL yeast transfer RNA. Aliquots should be stored at -20^0C or lower.

PCR Reaction Buffer: A 10-50 mM Tris-HCl solution (pH 8.3) containing 25 mM potassium chloride, 1.5 to 2.25 mM magnesium chloride, and 0.1 mg/mL gelatin. Aliquots should be stored at -20^0C or lower.

Isopropanol, stored at room temperature.

Ethanol, a 70-75% solution, stored at room temperature.

MuLV Reverse Transcriptase (4,000 U/mL), stored at -20^0C.

Dithiothreitol, 0.1 M solution. Aliquots should be stored at -20^0C or lower.

RNAsin (40,000 U/mL), or equivalent, stored at -20^0C.

Noniodet P-40, a 10% solution, stored at room temperature.

Taq DNA polymerase (2.5 units/mL), stored at -20^0C or lower.

Deoxynucleotide (dNTPs): A solution containing 25 mM each of deoxyguanosine (dGTP), deoxyadenosine (dATP), deoxycytosine (dCTP), deoxythymidine (dTTP). Aliquots should be stored at -20^0C or lower.

PCR primer "A'": A solution containing the oligomer
5'-TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "NE1'": A solution containing the oligomer
5'-CTTACTAACTTCTGTATGTCATTGACAGTCCAGCT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "B": A solution containing the oligomer
5'-GGATGGAAAGGATCACC-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^0C or lower.

PCR primer "215WT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGGT-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

PCR primer "215MUT": A solution containing the oligomer
5'-ATGTTTTTTGTCTGGTGTGAA-3'
at a concentration of 250 ng/µL. Aliquots should be stored at -20^{0}C or lower.

Water: Distilled grade or higher, stored at room temperature.

Wildtype Control DNA: A solution containing DNA from the HIV RT gene containing the wildtype (ACC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

Mutant Control DNA: A solution containing DNA from the HIV RT gene containing the mutant (TAC or TTC) sequence at Codon 215. Aliquots should be stored at -20^{0}C or lower.

TBE Buffer: A solution containing 0.089M Tris borate (pH 8.3) and 0.01M EDTA, stored at room temperature.

Agarose: Molecular-biology grade, stored at room temperature.

Molecular Weight Marker: 100 base-pair marker (or equivalent) for electrophoresis, stored at 4^{0}C.

Gel Loading Buffer: A solution of 30% glycerol containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, or equivalent, stored at 4^{0}C.

IV. SUPPLIES AND EQUIPMENT

1.5 mL Siliconized Microcentrifuge Tubes
PCR Reaction Tubes and Caps
Perkin-Elmer GeneAmp PCR 9600 Thermal Cycler (or equivalent)
Pipettors (20 µL, 200 µL, and 1000 µL)
Aerosol-resistant Pipette Tips
Vortex Mixer
Water Bath, Incubator, or Heat Block for 56^{0}C (±2^{0}C) and 99^{0}C (±2^{0}C)
Speed-Vac, or equivalent (optional)
Agarose Electrophoresis Apparatus
Camera
Ultraviolet light source
High Speed Film
Latex Gloves
Lab Coat

V. PROCEDURE

A. Sample Preparation.

1. Method A.

a. Pipette 0.2 to 1.0 mL of test sample into a 1.5 mL microcentrifuge tube.

b. Centrifuge the sample for 30 min at 24,000 g or 10 min at 125,000 x g.

c. Discard the supernatant, add 400 µL of Solution D, and vortex.

d. Add 10 µL yeast transfer RNA and 26 µL of sodium acetate. Vortex.

e. Add 400 µL of phenol and 80 µL of chloroform:isoamyl alcohol. Vortex.

f. Place the samples on ice for 10 min.

g. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

h. Transfer the upper aqueous layer to a new 1.5 mL microcentrifuge tube, and appropriately discard the lower organic layer.

i. Add 450 µL of isopropanol to each sample, vortex, and hold at -20^0C for at least 1 hour.

j. Centrifuge the samples for 15 min at 12,000 x g at 4^0C.

k. Discard the supernatant, and add 1 mL of 70-75% ethanol.

l. Add 500 µL of 70-75% ethanol and vortex.

m. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

n. Discard the supernatant, and add 0.5 mL of 70-75% ethanol.

o. Centrifuge the samples for 10 min at 12,000 x g at 4^0C.

p. Discard the supernatant, and add 0.2 mL of 70-75% ethanol.

q. Centrifuge the samples for 15 min at 12,000 x g at 4^0C.

r. Discard the supernatant.

s. Air dry the pellet or use a Speed-Vac, just to dryness (do not over dry).

t. Resuspend the dried pellet on 25 µL of Viral Lysis Buffer.

2. Method B.

a. Pipette 0.2 to 1.0 mL of test sample into a 1.5 mL microcentrifuge tube.

b. Centrifuge the sample for 30 min at 24,000 g or 10 min at 125,000 x g.

c. Discard the supernatant, add 800 µL of Tri-Reagent, and vortex.

d. Hold at room temperature for 3 min.

e. Add 160 µL of chloroform and vortex.

f. Hold at room temperature for 3 min.

g. Centrifuge at 12,000 x g for 15 min at 4^0C.

h. Transfer the upper aqueous layer to a new 1.5 mL microcentrifuge tube.

i. Add 160 µL of chloroform to the remaining organic layer, and vortex.

j. Centrifuge the organic layer at 12,000 x g for 15 min at 4^0C.

k. Remove the upper aqueous phase and add it to the previously collected aqueous layer (step h).

l. Appropriately discard the remaining lower organic phase.

m. Add 400 µL of isopropanol to the aqueous phase and vortex.

n. Hold the samples at -20^0C overnight.

o. Centrifuge the samples at 12,000 g for 14 min at 4^0C.

p. Discard the supernatant.

q. Add 1 mL of 70-75% ethanol and vortex.

r. Discard the supernatant.

s. Air dry the pellet.

t. Resuspend the pellet in 25 µL of Viral Lysis Buffer.

B. Reverse Transcription.

1. For each sample to be tested, prepare the following reaction mix:

5 µL PCR Reaction Buffer
1 µL dNTPs
1 µL primer NE1'
2 µL reverse transcriptase
2 µL RNAsin
2 µL 0.1M DTT
3.4 µL 10% NP-40
10.4 µL water

2. Dispense 25 µL of reaction mix into PCR reaction tubes.

3. Add the 25 µL of test sample into a reaction tube.

4. Incubate the reactions for 10 min at 25^0C, 30 min at 42^0C, and 5 min at 95^0C.

C. First Round PCR.

1. For each sample to be tested, prepare the following reaction mix:
5 µL PCR Reaction Buffer
1 µL dNTPs
1 µL primer A'
0.5 µL Taq polymerase
42.5 µL water

2. Mix the reaction mix thoroughly.

3. Pipette 50 µL of the test sample into the reaction tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
35 cycles of:	94^0C for 30 sec
	55^0C for 30 sec
	72^0C for 60 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples can be stored at 4^0C for 24 hr or -20^0C or lower for longer periods.

C. Second Round PCR.

1. For each sample to be tested, prepare the following reaction mixtures:

Wildtype:	10 µL PCR Reaction Buffer
	1 µL dNTPs
	1 µL primer B
	2 µL primer 215WT
	0.5 µL Taq polymerase
	81.5 µL water

Mutant:	10 µL PCR Reaction Buffer
	1 µL dNTPs
	1 µL primer B
	2 µL primer 215MUT
	0.5 µL Taq polymerase
	81.5 µL water

2. Mix the reaction mixes thoroughly and pipette 95 µL of each reaction mix to separate PCR reaction tubes.

3. Pipette 5 µL of the first round PCR product into a wildtype reaction mix tube and another 5 µL into a mutant reaction mix tube.

4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of:	95^0C for 90 sec
30 cycles of:	94^0C for 60 sec
	48^0C for 30 sec
	72^0C for 30 sec
1 cycle of:	72^0C for 10 min

5. At the completion of the cycling, the samples may be analyzed immediately or stored at 4^0C or lower.

D. Gel Electrophoresis.

1. Prepare a 3% agarose gel containing 0.5 µg/mL ethidium bromide in TBE.

2. Pipette 5 µL of Gel Loading Buffer into two microcentrifuge tubes.

3. Pipette 10-20 µL of each second-round PCR product into a tube and mix.

4. Load the samples into adjacent wells on the gel. Include 1 lane of 100 bp ladder per gel.

5. Run the gel in TBE for approximately 1 hr at 120V.

6. Remove the gel from the electrophoresis apparatus and photograph the gel under ultraviolet light.

VI. CALCULATIONS/DATA ANALYSIS

Samples which have a 210 base-pair product (band) in the wildtype reaction and do not have a 210 base-pair product in the mutant reaction are considered to have wildtype sequences at Codon 215. Samples which have a 210 base-pair product in the mutant reaction and do not have a 210 base-pair product in the wildtype reaction are considered to have mutant sequences at Codon 215. If a 210 base-pair product is detected in both reactions, the first round PCR product is diluted 1:1000, and 10 µL of the dilution is amplified in another second round PCR reaction. If a 210 base-pair product persists in the wildtype reaction but disappears from the mutant reaction, the sample is considered to have the wildtype sequence. If a 210 base-pair product persists in the mutant reaction but disappears from the wildtype reaction, the sample is considered to have the mutant sequence. If a 210 base-pair product persists in both wildtype and mutant reactions, the sample is considered to have a mixture of wildtype and mutant sequences at Codon 215.

VII. QUALITY CONTROL

For all amplifications, a reaction mix control consisting of reaction mix with water instead of test sample is required. The sample results for a given assay are invalid when a 210 base-pair band is detected in this control.

The second round PCR requires control reactions consisting of reaction mix with first-round PCR products of known wildtype and mutant viruses. These reactions will validate the ability of the wildtype and mutant reactions to accurately detect wildtype or mutant DNA in the test samples. The sample results for a given assay are invalid when either of these controls do not yield the appropriate 210 base-pair product.

VIII. PROCEDURE NOTES

A. Work areas in which the different PCR steps are performed should be geographically separated. At least, the room in which the second-round PCR product is analyzed should be separate from the areas in which reaction mixes and samples are prepared. A better

arrangement is to have a separate room for product analysis, sample preparation, and reaction mix preparation.

B. Good laboratory technique is essential to the proper performance of this assay. Extreme care should be taken to avoid the contamination of reagents with samples, controls, or PCR product and RNase in the sample preparation steps. Any reagent suspected of contamination should be discarded.

C. All pipettors, tips, reagents, and lab coats should be dedicated to and kept in only one PCR area (e.g., pipettors for loading gels cannot be used to prepare samples or reagents; lab coats worn during loading gels should not be worn into the reagent preparation area).

D. Pipette tips with aerosol barriers should be used during all steps.

E. PCR reaction trays (if used) should be soaked in bleach, rinsed with water, and dried before being used for another set of reactions.

F. Operators should pay close attention when transferring first-round PCR product into second-round reactions. Transferring an inadequate volume to one of the two reactions (wildtype or mutant), completely missing one of the two reactions, or confusing the identity of sample tubes can provide incorrect results without the mistake being obvious.

IX. LIMITATIONS

A. As with any PCR-based test, accurate detection of the Codon 215 mutation depends on extreme care being taken by laboratory technicians in handling reagents and performing the test, to ensure that any results obtained are derived from the patient's specimen and not from external sources.

B. Samples collected in heparin-containing tubes, vials, or equipment interfere with PCR reactions, which often results in no amplification. To ensure the greatest possibility of obtaining results, specimens should be collected in non-heparin-containing vessels.

C. The presence or absence of Codon 215 mutations cannot independently predict or determine the course of HIV infection. The results of this test should only be interpreted in conjunction with other laboratory and clinical data available to the clinician.

X. REFERENCES

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